CU-Boulder Protocol

Nebivolol and Endothelial Regulation of Fibrinolysis
08/25/2015

2. Description of Project

Purpose. The specific aim and hypothesis associated with the present research protocol are:

Experimental Aim: To determine, *in vivo,* the effects of nebivolol on the capacity of the vascular endothelium to release t-PA in adult humans with elevated blood pressure.

Hypothesis. *We hypothesize that nebivolol will improve endothelial t-PA release in adult humans with elevated blood pressure to a greater extent than either metoprolol or placebo. We further hypothesize that the improvement in the capacity of the vascular endothelium to release t-PA with nebivolol is mediated, in part, by the compound's antioxidant properties.*

Results supporting this hypothesis will provide important clinical data and novel mechanistic insight into the unique antihypertensive and cardioprotective effects of nebivolol.

Background:
The development of fibrous plaque and the resulting ulceration and subsequent thrombus formation within the lumen of an artery is recognized as a precipitating cause of atherothrombotic events such as angina, myocardial infarction, thrombotic stroke, and sudden cardiac death (47). The hemostatic mechanism responsible for the proteolytic degradation of intravascular fibrin deposition is the fibrinolytic system. When activated, this enzymatic pathway degrades fibrin within the developing thrombus, thus preventing arterial occlusion and interruption of blood flow. Both clinical and epidemiological data indicate that reduced endogenous fibrinolytic activity is a major contributor to the development, progression, and severity of atherothrombosis (17, 18, 52, 53, 57, 59, 68). Endothelial cells are the principal site of synthesis and release of tissue-type plasminogen activator (t-PA), the primary plasminogen activator in fibrinolysis, and a major producer of its biological inhibitor plasminogen activator inhibitor-1 (PAI-1) (40). t-PA is the key enzyme in initiating an endogenous fibrinolytic response due to its ability to preferentially activate plasminogen on the surface of developing thrombi (27). Recent data indicate that it is the capacity of the endothelium to release t-PA rapidly and acutely from intracellular storage pools (25, 48) and *not* circulating plasma fibrinolytic concentrations, that determines the efficacy of endogenous fibrinolysis because thrombolysis is much more effective if active t-PA is readily available and incorporated during, rather than after, thrombus formation (5, 28). Thus, the capacity of the endothelium to release t-PA is considered to be a major endogenous defense mechanism against thrombosis (9, 44, 54). It has been suggested that impaired fibrinolytic capacity may contribute to increased cardiovascular risk with elevated blood pressure (24), i*dentifying an important endothelial target for primary and secondary prevention of atherothrombosis in prehypertensive and hypertensive individuals.* Very little clinical data are available regarding the effects of antihypertensive agents on endothelial control of the fibrinolytic system.

Nebivolol, a third generation beta-blocker with high selectivity for b_1-adrenergic receptors, has proven to be highly effective in treating hypertension (3, 14, 39). A distinguishing feature of nebivolol from other beta-blockers is its hemodynamic profile, specifically the unique ability to enhance both basal and stimulated nitric oxide (NO) release resulting in peripheral vasodilation, improved endothelial function and increased myocardial compliance (19, 38, 56, 74). However, the favorable vascular effects of nebivolol that contribute to its blood pressure lowering action, and possible cardioprotectant effects, may

not be limited to endothelial vasomotor function. Indeed, there are *in vitro* data to suggest that nebivolol may have favorable effects on the fibrinolytic system, but there is currently no *in vivo* clinical evidence that treatment with nebivolol improves vascular endothelial t-PA release. *This represents a critical void in understanding the potential unique antithrombotic effects of nebivolol.*

Elevated blood pressure, aside from aging, is the most prevalent cardiovascular disease risk factor. Sub-optimal blood pressure (BP≥130/80) affects more than 100 million people in the United States and more than 1 billion people worldwide (55). Aggressive reduction of blood pressure can significantly reduce cardiovascular risk and acute thrombotic events (16, 63). Nebivolol is a well tolerated, highly effective antihypertensive agent with minimal adverse metabolic effects making it a useful first-line monotherapy treatment option for the management of patients with mild to moderate essential hypertension (19, 38, 42, 56, 74). The hemodynamic effects of nebivolol, specifically its NO enhancing property, differentiate it from traditional beta-blockers (38, 41). While the importance of the NO system to vasoregulation and cardiovascular health is well established, so too is the role of endothelial fibrinolytic regulation in the pathogenesis of hypertension-related thrombotic risk (34). Another potential area of differentiation regarding the cardiovascular benefit of nebivolol compared with other beta-blockers may be its effect on endothelial fibrinolytic function. Considering endogenous fibrinolytic potential is determined at the vascular wall, understanding the modulatory effects of nebivolol on endothelial release of t-PA would provide further insight into unique vascular, and thromboprotective, effects of nebivolol. *The results of the proposed study will provide new and clinically important information regarding the effects of nebivolol on endogenous fibrinolytic potential in adults with sub-optimal blood pressure.*

2. Methodology:

I.) Subjects. Subjects will be men and women of all races and ethnic backgrounds aged 45-65 years. The rationale for studying this age group is based on epidemiological data showing that the prevalence of suboptimal blood pressure is highest in this age-range (26). Subjects will be normotensive defined as resting blood pressure <120/80 mm Hg and prehypertensive/hypertensive defined as resting systolic blood pressure ≥125 mmHg and/or diastolic blood pressure ≥80 mmHg (15). The rationale for including adults with prehypertension is that this is a rapidly expanding segment of the population who are likely to develop clinical hypertension (15, 24, 26, 46, 66) and who already exhibit many of the vascular abnormalities common with hypertension (75).

All of the women in the study will be postmenopausal and not receiving hormone replacement therapy (HRT) currently or in the preceding 3-year period. Our rationale for this approach is as follows. First, if pre-, peri-, and postmenopausal women all were included in this cohort the widely varying circulating reproductive hormone levels (which may have profound effects on insulin sensitivity as well as endothelial vasomotor function) could introduce substantial variability into this group making it difficult to identify main effects of obesity/insulin resistance (if present). Second, with respect to HRT use, the modes of application, the dosage of estrogen and whether or not it is combined with progesterone, the number of years of use, and many other factors can vary widely among individual women, adding significant inter-subject variability to the data.

To establish blood pressure status, resting blood pressure measurements will be performed in the sitting position between 8 a.m. and 10 a.m. on at least 2 separate days one week apart to establish both normal and sub-optimal blood pressure (resting systolic blood pressure ≥125 mmHg and/or diastolic blood pressure ≥80 mmHg). Subjects will not have ingested caffeine-containing beverages prior to all blood pressure measurements. The recordings will be made under quiet, comfortable ambient (~24°C) laboratory conditions. To avoid any possibility of investigator bias, measurements will be made with a semi-automated device (Dinamap, Crtikon, FL) that uses an oscillometric technique over the brachial

artery. Recordings will be made in triplicate in the upright sitting position. All measurements will conform strictly to American Heart Association guidelines as established by the Council for High Blood Pressure Research (49).

Candidates who smoke (currently or in the past 7 years), report more than low-risk alcohol consumption as defined as no more than 14 standard drinks/wk and no more than 4 standard drinks/day for men and 7 standard drinks/wk and 3 standard drinks/day for women (a standard drink is defined as 12 ounces of beer, 5 ounces of wine, 1½ ounces of 80-proof distilled spirits) (71) or who are taking cardiovascular-acting (i.e. statins) medications will not be eligible.

Subjects will be free of overt chronic diseases as assessed by: a) medical history; b) physical examination; c) electrocardiogram and BP at rest; d) blood chemistries and lipid and lipoprotein evaluation; and e) fasting plasma glucose <126 mg/dL (2). In addition potential candidates with a resting heart rate of $\leq$ 50 beats/minute will be excluded. All fasting plasma lipid and lipoprotein, glucose and insulin concentrations will be determined by the UC-Boulder Clinical and Translational Research Center (CTRC) core laboratory using conventional methods (1, 10, 30).

Lastly, candidates will be sedentary as determined from the Stanford Physical Activity Questionnaire (<35 kcal/wk; (58)) and will not have engaged in any program of regular physical activity for at least 1 year prior to the study. The nature, purpose, and risks of all procedures and protocols will be explained to each subject prior to obtaining their written informed consent.

General Experimental Design:

The proposed study will employ a 3-month randomized, double-bind placebo controlled single site trial to determine the effects of nebivolol on the capacity of the vascular endothelium to release t-PA in adults with suboptimal blood pressure. An initial telephone screening will be used to ensure that interested candidates satisfy the basic criteria of the study (see above). Potential candidates, after providing written informed consent, will be invited to undergo laboratory screening to assess blood pressure status. Candidates who successfully meet the blood pressure requirements and complete the screening process will be enrolled in the study. The subjects will then undergo baseline testing and assessment of endothelial t-PA (described in detail below under "Measurements and Procedures"). Drs. DeSouza and Stauffer have successfully recruited and studied human subjects across the adult age-range with varying levels of blood pressure in the Boulder-Denver metropolitan area since 1995 (20, 22, 23, 32, 33, 60-62, 64, 73).

Intervention. Eligible subjects will be *randomly* assigned (computer generated assignment) to one of three treatment groups: nevibolol (5 mg/day), metoprolol (100 mg/day) or placebo (control condition). Subjects will participate in their respective treatment for a period of 12 weeks (~3 months). During this 12-week period measurements of the key outcome variables will be made at baseline and 12 weeks. Before randomization subjects will be reminded that the purpose of the study is to determine the effects of nebivolol on ET-1 vasoconstrictor tone and fibrinolytic capacity and that they will be randomly assigned to one of the three treatments.

The subjects will be instructed to take their medication (nebivolol, metoprolol or placebo) in the morning, except on the morning of a testing session, and not to take any other medications during the course of the study. Subjects will return to the UC-Boulder Clinical and Translational Research Center (CTRC) every two weeks to have their body weight and blood pressure measured, receive their next allotment of pills and to meet with a study research assistant to discuss any problems he/she may be experiencing. Adherence to each treatment will be documented by bi-monthly pill counts. The CTRC pharmacy will

dispense all pills.

Dr. Stauffer will oversee the randomization of subjects and will not be blinded to treatment group assignment of the subjects.

Drug Tapering: At the completion of the study, or if a subject withdraws from the study at any time, the doses of nebivolol and metoprolol will be tapered over a 3-day period. Nebivolol will be tapered as follows: 2.5 mg/day for 3 days then stop. Metoprolol will be tapered as follows: 50 mg/day for 1 day, 25 mg/day for 2 days then stop.

General Information for All Protocols. All protocols will be performed in the outpatient research protocol laboratories in the UC-Boulder CTRC providing the highest quality of technical and clinical support for the safe and successful performance of the proposed intra-arterial infusion protocols.

Experimental Hypothesis

Nebivolol will improve endothelial t-PA release in adult humans with elevated blood pressure to a greater extent than either metoprolol or placebo. We further hypothesize that the improvement in the capacity of the vascular endothelium to release t-PA with nebivolol is mediated, in part, by the compound's antioxidant properties.

Experimental Design and Strategy. To determine the effects of nebivolol on endothelial t-PA release *in vivo,* an isolated human forearm model will be used (11-13). This approach has the advantages of allowing for: (a) stimulation of local and rapid endothelial release of t-PA (by intra-arterial infusion of various vasoactive agents) without producing potential confounding systemic responses; (b) direct determination of endothelial t-PA release *in vivo* free of the confounding effects of hepatic clearance and shifts between free and complexed (with PAI-1) molecular forms; (c) the study of an intact viable vascular bed with preserved innervations, blood flow, and cell-to-cell interaction (7, 36, 37, 65); and (d) a peripheral measure of endothelial t-PA release that correlates strongly and positively with t-PA release in the coronary circulation (45)**.**

Study Methods. Subjects: Middle-aged and older male and female adults (see above under "Subjects"). Preliminary Measurements (separate day from main protocol): DXA measures of body mass and composition, and dietary analysis (see above under "Measurements and Procedures"). Main Protocols: Assessing Endothelial t-PA Release: The brachial artery will be catheterized as described above under "Measurements and Procedures". An intravenous catheter will then be placed in an antecubital vein of the same arm. We (32, 33, 72, 73) and others (7, 36, 37, 65) have shown that brachial artery catheterization and intravenous catheterization in the antecubital region of the same arm is safe and well tolerated by adult human subjects. The experience of the investigative team and CTRC environment will provide the highest level of technical and clinical support for the safe and successful performance of the proposed arterial and venous catheterizations.

After arterial and venous catheterization, instrumentation, and establishment of steady-state baseline conditions and prior to beginning the infusion protocol, blood will be drawn (5 mL) from the antecubital vein for basal fibrinolytic measures. Following the measurement of baseline FBF and FBF to acetylcholine (doses: 4.0, 8.0, 16.0 μg/100 mL tissue/min), and at the end of each dose of bradykinin and sodium nitroprusside, blood will be drawn simultaneously from the brachial artery and antecubital vein of the experimental arm to measure local release of fibrinolytic factors. Bradykinin was selected to stimulate endothelial t-PA release based on its specificity and effectiveness at eliciting local and rapid endothelial t-PA release in adult humans (6, 7). Bradykinin will be infused at 12.5, 25, 50 ng/100 mL tissue/min and

sodium nitroprusside at 1.0, 2.0, 4.0 μg/100 mL tissue/min for 5 min at each dose. The concentrations of bradykinin have been shown to elicit significant dose-response increases in the net release of t-PA without affecting net release of PAI-1 (8). Fibrinolytic determinations in response to sodium nitroprusside are required to establish that any observed differences in local endothelial release of t-PA to bradykinin are not due to increased blood flow related shear stress (8, 31, 35). Each drug will be administered in random order after allowing sufficient time for FBF and plasma fibrinolytic concentrations to return to resting levels (30 minutes) (32, 33, 72, 73). Net endothelial release or uptake of t-PA and PAI-1 (both antigen and activity levels) at each time point will be calculated as the product of the arteriovenous concentration gradient and the infused forearm plasma flow. Arteriovenous concentration gradients for both t-PA and PAI-1 antigen and activity for each subject (at each time point) will be determined by subtraction of the values measured in simultaneously collected venous and arterial blood samples. A positive difference indicates a net release and a negative difference, a net uptake. Forearm plasma flow will be calculated from FBF and arterial hematocrit corrected for 1% trapped plasma. Thus, net release (or uptake) will be calculated using the following equation (7, 37).

$$\textbf{Net Release} = (C_V\text{-}C_A) \text{ X } (\text{FBF X } [101\text{-Hematocrit}/100])$$

$(C_V\text{-}C_A)$ = arteriovenous concentration gradient
C_V = venous concentration C_A = arterial concentration

Intra-Arterial Administration of Vitamin C. Acute intra-arterial infusion of the potent antioxidant vitamin C will be used to assess the vascular effects of oxidative stress on bradykinin-stimulated endothelial t-PA release before and after each intervention. Our rationale for selecting vitamin C over other antioxidants such as vitamin E is as follows. First, vitamin C is the main water-soluble antioxidant in human plasma (29). Second, it effectively scavenges superoxide and other reactive oxygen species and spares other endogenous antioxidants from consumption (4, 51). Third, it plays a key role in the regulation of intracellular redox state through its interaction with glutathione (43, 76). Fourth, vitamin C has been used successfully to demonstrate that oxidative stress contributes to endothelial fibrinolytic dysfunction in a number of pathologies, including hypertension (72).

After allowing sufficient time (~20 minutes) for FBF to return to baseline following the initial infusions of bradykinin, sodium nitroprusside and vitamin C will be infused at a constant rate (12 mg/100 mL tissue/min) for 5 minutes. The vitamin C dose was selected because it has been shown to both protect human plasma from free radical-mediated lipid peroxidation (29) and improve endothelium-dependent vasodilation and endothelial t-PA release in conditions associated with oxidative stress (50, 67, 69, 70, 72). The vitamin C infusion will be maintained at the same rate while the bradykinin and sodium nitroprusside dose-response curves are repeated in the same order as performed earlier. Net endothelial release rates of t-PA and PAI-1 (antigen and activity) will be determined at time 0 and 5 minutes of vitamin C infusion and after each dose of bradykinin and sodium nitroprusside in the presence of vitamin C. A 15-minute washout period will be provided between each dose response curve (72). The acute vitamin C protocol will be performed at baseline and after each intervention.

Statistical Analysis. The proposed statistical approach was chosen to take advantage of the randomized study design with repeated measures. Differences in the FBF responses and t-PA and PAI-1 responses to each vasoactive agent (bradykinin, sodium nitroprusside, bradykinin+vitamin C, sodium nitroprusside+vitamin C) will be determined by a mixed-effects regression model. We chose a mixed-effect model over a repeated measures ANOVA to accommodate any missing data (due to drop-out) and to allow us to model a more realistic covariance structure. The model will include factors for treatment

condition (indicator variables for each treatment condition) and any covariates such as baseline blood pressure, age, gender that were not balanced under the randomized design (to be tested using one-way ANOVA prior to mixed model analysis). To test for any trends over time, we may also include a term for the time of measurement (and interaction term of time with treatment) to determine if there is any impact of the time of measurement on measures of FBF. We will initially model the covariance structure of the repeatedly measured FBF and fibrinolytic responses assuming an unstructured pattern and test whether more restrictive covariance assumptions (i.e. exponential, compound symmetry) are more appropriate. Given the design, treatment conditions and assessment protocols, we anticipate the missing data (due to subject dropout) to be missing at random (MAR). In other words, for example, we do not expect the initial treatment condition to influence whether a subject opts to continue with the study. Nevertheless, we plan to explore this issue by using all available baseline information to determine whether dropout is associated with any of the important covariates (baseline blood pressure, medication use and age) using logistic regression. However, as emphasized previously, we do not anticipate encountering missing data that are not missing at random (NMAR), given the treatment conditions under study. Assuming missing data that are MAR, an "intent-to-treat analysis" would yield valid inference. However, this approach would come at a cost of reduced statistical efficiency. Therefore, we will also employ Schafer's multivariate normal imputation approach as implemented in SAS v9.1 (SAS Institute Inc.; *proc MI* - MCMC Method and *proc MIANALYZE*). According to a recent simulation study, this multiple imputation approach performs well and generates more reliable information than complete-case analysis. Results from the complete-case analysis will be compared with that of the multiple imputation analysis to ensure consistency of the regression parameters. To account for multiple testing, the Bonferroni-correction will be used to adjust the significance level appropriately.

Sample Size Calculations. All power calculations were performed using nQuery Advisor 6.0 statistical software (Statistical Solutions, MA). Sample sizes were determined based on data from the P.I.'s laboratory and available published results of comparable treatment-related changes in endothelial t-PA release (61, 72, 73). Sample sizes were calculated based on 80% power at an alpha level of 0.05. This power value is equivalent to a probability of <0.20 of committing a type-II error. Power calculations yielded sample size of 18 subjects per treatment group (nebivolol, metoprolol, placebo) based on effect sizes of 0.74. This sample size (N=54; n=18/group) should be sufficient to determine clinically and physiologically significant differences between treatment groups if present. To account for an estimated 20% dropout rate, we will recruit 22 subjects per group.

II.) Recruitment: Subjects will be recruited from the greater Boulder/Denver area. It is important to note that the investigative team has been conducting cardiovascular research Boulder for over 15 years. During this time we have established a recruiting network for middle-aged and older adults. For example, our laboratory is supported by the City of Boulder Aging Services and we have access to senior centers in Boulder. Recruiting mechanisms include flyers posted around the community and from senior-emphasis organizations (see attached flyer).

III.) Copies of Surveys, Questionnaires, or Interview Schedules: Copy of Stanford Physical Activity Questionnaire will be submitted electronically.

IV.) Description of Procedures: All procedures will be performed at the CTRC unless otherwise stated. Estimated time of each procedure is provided in **bold.** The order in which the following procedures will be performed, in separate experimental sessions, is provided below under "Sequence of Measurements".

1) *Telephone Screening/Subject Information (performed by laboratory personnel at the Integrative Vascular Biology Laboratory located in Carlson Gymnasium).* When interested candidates voluntarily call the laboratory (after seeing our advertisements or through word of mouth) for more information on

the proposed study and once we have obtained verbal consent according to prescreen consent form they will be asked general questions regarding their, age, physical activity status, medical history to determine whether they satisfy the basic criteria for the study. Subjects who meet the basic criteria for the study will be invited to come to the CTRC to discuss the study in more detail, have the informed consents explained to them, and to provide written informed consent for blood pressure screening, and if appropriate, to participate in the study. Subjects who provide written informed consent will be scheduled to undergo screening and testing procedures at the University of Colorado CTRC. For subjects who do not meet the inclusion criteria all clinical screening information will be kept for a period of 10 years. All other information will be destroyed. **Total time: 60 minutes.**

2) *Physical Examination and Resting ECG.* A physician will perform a standard medical exam (like an office visit with a doctor) including vital signs such as heart rate and blood pressure as well as examination of ear, nose, throat, eye exam, and listen to your lungs and heart. **Total time: 30 minutes.**

3) *Body Composition* or body fat percentage will be measured non-invasively by skinfold thickness and by Dual Energy X-ray Absorptiometry or DXA. This test involves subjects lying on a padded table while a small probe, that emits energy to measure tissue density, passes over their body. This test will be performed before and after each intervention. **Total time: 30 minutes.**

4) *Blood Pressure* will be measured non-invasively in the laboratory by the standard cuff technique (i.e., as in a Doctor's office) and using an automated blood pressure monitor. **Total time: 20 minutes.**

5) *Daily Physical Activity* will be estimated using a standardized questionnaire. The questionnaire will be administered via personal interview with subject by a trained exercise physiologist associated with the CTRC. **Total time: 30 minutes.**

6) *Dietary Recall Records* will be used to determine total daily caloric intake and the amounts of carbohydrates, protein, and fat in subject's diets. Subjects will be instructed on how to complete their food records by the CTRC bionutritionist who will also review all records. **Total time for dietary instruction: 45 minutes.**

7) *Blood Draw to Measure Blood Chemistries and Risk Factors for Cardiovascular Disease* in order to measure well established risk factors for cardiovascular disease (such as blood cholesterol, glucose and insulin levels, oxidative markers, inflammatory cytokines, microparticles and microRNA) and to screen for disease subjects will be asked to fast overnight for 12 hours prior to the blood draw. A small needle will be inserted into a vein in the forearm to collect a sample of blood (~ 50 mL) for these risk factor determinations. **Total time: 15 minutes.**

8) *Forearm Blood Flow* will be measured by placing a flexible rubber cuff around the forearm while periodically inflating and deflating two blood pressure cuffs (one around the upper arm and one around the wrist). Flow will also be measured in response to the local infusion of small safe quantities of vasoactive drugs (bradykinin, sodium nitroprusside and vitamin C) into the brachial artery via an indwelling catheter. Bradykinin and sodium nitroprusside will cause blood flow to the forearm to increase. Note, a licensed medical person will place all indwelling arterial catheters and administer all vasoactive drugs. **Total time: 4.0 hours.**

9) *Fibrinolytic Factors.* Blood samples for determination of fibrinolytic factors will be collected in chilled tubes containing 0.45 mol/L sodium citrate buffer, pH 4.3 (Stabilyte, Biopool AB; final dilution volume 1:10) and stored as previously described by the P.I. (31, 32, 61). Arterial and venous plasma concentrations of t-PA and PAI-1 antigen and activity will be determined by enzyme immunoassay (31).

Reliability and reproducibility of these assays in our laboratory have been reported (21). These analyses will be performed in the Department of Integrative Physiology Core Biochemistry Laboratory. Total amount of blood to be collected is ~100 mL.

Study Sessions: Table 1 provides an overview of the study sessions associated with the study and the measurements to be performed at each session.

Session Screening and Experimental	Measurement *(All measures performed at the UC-Boulder CTRC)*	Time
1 (Screening)	Blood Pressure Resting ECG and Physical Examination Blood Draw	20 min 30 min 15 min
2 (Experimental)	Body Composition Daily Physical Activity Dietary Recall Records	30 min 30 min 45 min
3 (Experimental)	Assessment of Endothelial Fibrinolytic Capacity	4.0 hrs
4 (Experimental)	Blood Pressure Body Weight	30 min
5 (Experimental)	Blood Pressure Body Weight	30 min
6 (Experimental)	Blood Pressure Body Weight	30 min
7 (Experimental)	Blood Pressure Body Weight	30 min
8 (Experimental)	Blood Pressure Body Weight	30 min
9 (Experimental)	Blood Pressure Body Weight	30 min
10 (Experimental)	Body Composition Daily Physical Activity Dietary Recall Records	30 min 30 min 45 min
11 (Experimental)	Assessment of Endothelial Fibrinolytic Capacity	4.0 hrs

3. Benefits and Risks. Indirect Benefits: Subjects will receive the following benefits: 1) medical information-- a medical history, physical examination, blood pressure readings, blood levels of glucose and insulin, blood fats, and resting electrocardiograms free of charge; 2) physical information-- measurement of body fat and bone density levels free of charge; and 3) financial compensation—subjects will receive $40.00 for each experimental session completed (maximum $400 for participating in the study).

Risks: The risks associated with the experimental protocols include:

1. Body Composition: there is a small amount of radiation exposure (0.04 mRem) associated with the DEXA scan. This amount of exposure is less than the amount that a subject would receive on daily basis living in Colorado. This study will expose a subject to radiation in addition to the natural background radiation a subject would receive each day. The more radiation a subject receives over the course of their life, the greater the risk of having cancerous tumors or inducing changes in genes. The radiation in this study is not expected to greatly increase these risks, but the exact increase in such risks is unclear.

2. Venous Catheter Risks: a hollow needle/plastic tube will be placed in your arm for taking small blood samples, giving fluids, or infusing glucose and insulin (intravenous glucose tolerance test). This will be left in for as long as 4 hours. When the needle goes into a vein, it hurts for a short time. Also there will be the minor discomfort of having the needle/plastic tube taped to the subjects arm. In about 1 in 10 cases a small amount of bleeding under the skin will produce a bruise. The risk of a blood clot forming in the vein is about 1 in 100, while the risk of infection or significant blood loss is 1 in 1,000. There is a side effect/risk of participants fainting as a result of placing the venous catheter and/or drawing blood from the vein.

3. Arterial Catheter Risks: placing a catheter into an artery causes some discomfort/pain, however, subjects will receive a small amount of numbing medication to lessen this discomfort/pain. In about 1 in 10 cases a small amount of bleeding under the skin will produce a bruise. Very rarely (1 in 4,000) damage may occur to the artery which can result in decreased blood flow to the forearm or hand caused by a blood clot or artery spasm. Although rare, these conditions can be serious if they develop and may require surgery to correct. Tests involving arterial catheters will be performed twice – before and after the 3-month intervention. There is a side effect/risk of participants fainting as a result of placing the arterial catheter and/or drawing blood from the artery.

4. Infusion of Vasoactive Drugs: infusion of bradykinin and sodium nitroprusside cause only local changes in forearm blood flow. Since low doses of these drugs will be used there is little or no effect on the rest of the body. There is no pain or discomfort associated with the drug infusions. The only significant risks associated with the infusion of the drugs are: a small drop or increase in blood pressure ($\pm$5-7 mm Hg); very slight chance of chest tightness, light-headedness, headache, and fainting; slight chance of an allergic reaction. There are no documented cases of serious events such as heart attack or stroke with the small doses of the drugs used in this procedure. The investigators have FDA approval to for this research (FDA IND#s are provided in the electronic application). **Drs. DeSouza and Stauffer are currently conducting UC-IRB approved research protocols involving the above vasoactive drugs at the doses outlined in this protocol.**

5. Vitamin C: The intra-arterial dose (24 mg/min) used for this study has been shown to be safe and without side effects.

6. Nebivolol: The most common adverse reactions that may occur with nebivolol are headache, nausea, diarrhea, tiredness, dizziness, slow heart rate, low blood pressure (hypotension) insomnia and leg

swelling. Subjects will be monitored closely throughout the study. Should they experience any of these symptoms or other symptoms that they think might be associated with the treatment they are instructed to call the CTRC at 303-735-2304 or after hours at 303-206-6339 (nurse pager). The nurse may contact Dr. Brian Stauffer if necessary. Drug package insert for nebivolol has been submitted electronically. Subjects will be asked to not stop taking their treatment pills without consulting with a CTRC nurse or the study investigators.

7. Metoprolol (TOPROLOL-XL [extended release]): The most common adverse reactions that may occur with metoprolol are tiredness, dizziness, slow heart rate, diarrhea, shortness of breath, pruritus (itching), rash, depression, and low blood pressure (hypotension). Subjects will be monitored closely throughout the study. Should they experience any of these symptoms or other symptoms that they think might be associated with the treatment they are instructed to call the CTRC at 303-735-2304 or after hours at 303-206-6339 (nurse pager). The nurse may contact Dr. Brian Stauffer if necessary. Drug package insert for metoprolol has been submitted electronically.

There are no alternative methods that would provide the same type of accuracy of information as the state-of-the-art procedures proposed in this application. It is important to note that all invasive procedures will take place at the University of Colorado CTRC under the supervision of Dr. Stauffer or the CTRC medical staff. Furthermore, emergency medical care will be immediately available during all testing. There are no risks to the investigators.

4. Assurance of Confidentiality. All data collected on individual subjects will only be viewed by the investigative team involved in the project (i.e., the Principal Investigator, Research Assistant, and Pre- and Post doctoral Research Fellows, and CTRC staff), although these data will be provided to the subject. The individual data for each subject will be analyzed and stored by code, and at no time will any individual data point be identified with a particular individual subject. The data will almost exclusively be presented as average responses. All data will be stored on hard and compact disk using the aforementioned codes. All data and information regarding the study will be stored in a secure (always-locked) filing cabinet in the Integrative Vascular Biology Laboratory in the Carlson building on the CU-Boulder campus. Data will be kept for 10 years after the study is completed then erased.

5. Consent Form. See Attached.

6. Investigator's Qualifications. The principal investigator for this project is Christopher A. DeSouza, Ph.D. Dr. DeSouza is Professor in the Department of Integrative Physiology. Dr. DeSouza received his doctoral training in the Department of Kinesiology, University of Maryland and Division of Gerontology at the Baltimore Veterans Affairs Medical Center in Baltimore Maryland and has been conducting research in the area of vascular function and aging in humans for the past 15 years. Dr. Stauffer is a board certified cardiologist and an Associate Professor of Cardiology in the Department of Medicine at the UC-Denver. Dr. Stauffer has extensive experience conducting studies involving vasoactive drugs such as the ones proposed herein. The medical staff at the CTRC are appropriately trained and have experience safely administering the procedures outlined in this protocol.

7. Publications employing the use of bradykinin and sodium nitroprusside in different study populations.

Bradykinin and Sodium Nitroprusside References:

Cardillo C, CM Kilcoyne, AA Quyyumi, RO Cannon, and JA Panza. Selective defect in nitric oxide synthesis may explain the impaired endothelium-dependent vasodilation in patients with essential hypertension. *Circulation*: 851-856, 1998.
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